UNIVERSITI PUTRA MALAYSIA

MOLECULAR DETECTION, SEROTYPING AND PHYLOGENETIC ANALYSIS OF Haemophilus parasuis IN PENINSULAR MALAYSIA

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MOLECULAR DETECTION, SEROTYPING AND PHYLOGENETIC ANALYSIS OF *Haemophilus parasuis* IN PENINSULAR MALAYSIA

By

LEE CHEE YIEN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Veterinary Science

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DEDICATION

My family,

Father and Mother

who have constantly love me and support me

Brother, Sister

who have kept me entertained all this time

Frankie Lau

who never complain about me

My PG comrades

Li Ping, Vynter, Michelle, Vivian, Daniel, Zi Herk, Rathiy, Peggy, Xiao Fen

Friends

And to all those who have made me who I am today.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Veterinary Science

MOLECULAR DETECTION, SEROTYPING AND PHYLOGENETIC ANALYSIS OF *Haemophilus parasuis* IN PENINSULAR MALAYSIA

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Chairman : Associate Professor Ooi Peck Toung, DVM, PhD
Faculty : Veterinary Medicine

Haemophilus parasuis infections, what is known to cause Glässer's disease has been a nuisance for all farms. The lack on the information of *H. parasuis* serotype complicates vaccination as current available commercial vaccines are serotype-based and efficacy is serotype-dependent. Hence, this study carried the objectives to detect *H. parasuis* in clinical samples from weaner pigs in Peninsular Malaysia with conventional polymerase chain reaction (PCR) assay; to detect *H. parasuis* serotype 4, 5 or 12 and 13 using multiplex PCR as these serotypes were prevalent in other countries; to detect the outer membrane protein P2 (*OMPP2*) gene and further investigate the phylogenetic relatedness of Peninsular Malaysia's *H. parasuis* with reference strains. Throughout October 2016 to June 2018, samples were collected from nine farms from northern, seven farms from central and four farms from southern regions of Peninsular Malaysia using convenience sampling method. A total of 87 pigs was sampled and from there, 323 samples were collected. The number of pigs and samples collected in each farm varied depending case availability and gross post mortem manifestation. Samples collected included lung, brain, bodily fluids and fibrins of various sites. Samples were screened for *H. parasuis* using conventional PCR assay and positive samples were serotyped by multiplex PCR. Finally, representative samples of every states and serotypes were subjected to conventional PCR assay for *OMPP2* gene detection; followed by sequencing and phylogenetic analysis on sequences obtained. Using conventional PCR assay, 58/87 (66.7%) of the pigs sampled were positive of *H. parasuis*. The region with highest detection of *H. parasuis* in pigs was the southern region (4/5), followed by northern region (12/18) and central region (42/64). Pigs apparent of *H. parasuis* infection (Category A, B and C) had a higher detection percentage compared to those that were unapparent (Category D, E and F). Among all of the sample types collected, the highest detection percentage was obtained in the pleural fibrin samples (85.7%).

Generally, multiplex PCR assay on *H. parasuis*–positive samples revealed that serotype 4, 5 or 12 and 13 were detected with predominance order of NT, 5 or 12 and followed equally by serotype 4 and 13. Furthermore, comparison of the partial *H. parasuis OMPP2* nucleotide sequence of 15 Malaysian isolates and 16 reference strains showed Malaysia isolates were closely related to China strains. There was no direct correlation between serotype and *OMPP2* gene. Moreover, phylogenetic analysis of *OMPP2* gene revealed that majority of Peninsular Malaysia isolates was found to belong to *H. parasuis* genetic type III group. In conclusion, *H. parasuis* and the three important serotypes 4, 5 or 12 and 13 were detected using PCR technique. The phylogenetic relatedness based on the *OMPP2* gene and its correlation with serotype was identified. The *OMPP2* gene served to be an important virulence gene and future studies should focus on this gene. Equip with the knowledge of this study, more effective control measures could be designed and implemented to control *H. parasuis* infections in Peninsular Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains Veterinar

PENGESANAN MOLEKULAR, PENCIRIAN SEROTIP DAN ANALISA FILOGENI *Haemophilus parasuis* DI SEMENANJUNG MALAYSIA

Oleh

LEE CHEE YIEN

April 2019

Pengerusi : Profesor Madya Ooi Peck Toung, PhD
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Infeksi *Haemophilus parasuis*, atau dikenali sebagai penyebab penyakit Glässer's telah menjadi gangguan kepada semua ladang. Kekurangan maklumat mengenai serotip *H. parasuis* menyukarkan vaksinasi kerana vaksin komersial yang ada sekarang adalah berdasarkan serotip dan efikasinya adalah bergantung kepada serotip. Oleh itu, objektif kajian ini adalah untuk mengesan *H. parasuis* dalam sampel klinikal babi di Semenanjung Malaysia dengan kaedah PCR konvensional; mengesan serotip utama *H. parasuis* ia adalah serotip 4, 5 atau 12, dan 13 menggunakan kaedah PCR multipleks; mengesan gen protein selaput luar P2 (*OMPP2*) dan selanjutnya menyiasat Kerterkaitan filogeni *H. parasuis* pencilan Semenanjung Malaysia dengan pencilan rujukan. Sepanjang Oktober 2016 sehingga Jun 2018, sampel adalah dikumpul daripada sembilan ladang di kawasan utara, tujuh ladang di kawasan tengah dan empat ladang di kawasan selatan Semenanjung Malaysia menggunakan kaedah persampelan mudah. Sejumlah 87 babi telah dikumpul dan daripada itu, 323 sampel telah dikumpul. Jumlah babi dan sampel yang dikumpul adalah berbeza untuk setiap ladang bergantung kepada kes dan manifestasi lesi. Sampel yang dikumpul meliputi paru-paru, otak, cecair badan dan fibrin dari pelbagai organ. Sampel adalah dikesan untuk *H. parasuis* menggunakan esei PCR konvensional dan sampel positif adalah ditip menggunakan PCR multipleks. Akhirnya, sampel yang mewakili setiap negeri dan serotip adalah dikesan untuk gen *OMPP2* menggunakan PCR konvensional; diikuti dengan penjujukan dan analisa filogeni ke atas jujukan yang diperoleh. Menggunakan PCR konvensional, 58/87 (66.7%) babi yang disampel adalah positif kepada *H. parasuis*. Kawasan yang mempunyai pengesanan *H. parauis* yang tertinggi adalah kawasan selatan (4/5), diikuti dengan kawasan utara (12/18) dan kawasan tengah (42/64). Babi yang ketara dengan infeksi *H. parasuis* (Kategori A, B, C) mempunyai pengesanan yang

lebih tinggi berbanding babi yang tidak ketara (Kategori D, E, F). Antara semua jenis sampel yang dikumpul, peratus pengesanan yang tertinggi adala diperoleh daripada sampel fibrin pleura (85.7%). Keseluruhannya, esei PCR multipleks ke atas sampel positif *H. parasuis* menunjukan bahawa serotip 4, 5 atau 12 dan 13 adalah dikesan dengan turutan dominasi NT, 5 atau 12 diikuti dengan jumlah yang sama adalah serotip 4 dan 13. Tambahan pula, perbandingan 15 jujukan nukleotid separa gen *OMPP2 H. parasuis* pencilan Malaysia dengan 16 jujukan rujukan menunjuk bahawa pencilan Malaysia adalah berkait rapat dengan strain China. Tiada korelasi terus antara serotip dengan gen *OMPP2*. Selain itu, analisis filogeni mendedahkan bahawa majoriti strain Malaysia tergolong dalam jenis genetik III. Kesimpulannya, *H. parasuis* dan tiga serotip penting iaitu serotip 4, 5 atau 12 dan 13 telah dikesan menggunakan teknik PCR. Kerterkaitan filogeni berdasarkan gen *OMPP2* dan kolerasi dengan serotip telah dikenal pasti. Gen *OMPP2* berfungsi sebagai gen virulen yang penting dan kajian seharusnya menumpukan kepada gen ini. Berdasarkan pengetahuan dari kajian ini, langkah-langkah kawalan efektif boleh direkabentuk dan dilaksanakan untuk mengawal jangkitan *H. parasuis* di Semenanjung Malaysia.

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I certify that a Thesis Examination Committee has met on 18 April 2019 to conduct the final examination of Lee Chee Yien on her thesis entitled "Molecular Detection, Serotyping And Phylogenetic Analysis of *Haemophilus parasuis* in Peninsular Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Veterinary Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Veterinary Science. The members of the Supervisory Committee were as follows:

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I hereby confirm that:

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This is to confirm that:

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Signature:	
Name of Member of Supervisory Committee:	Professor Dr. Noordin Mohamed Mustapha

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%	Percent
>	More than
<	Less than
µl	Microliter
µM	Micromolar
℃	Degree Celsius
AFLP	Amplified fragments length polymorphism
AGD	Agar gel diffusion
AMR	Antimicrobial resistant
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
C	Cytosine
CDS	Coding sequence
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EMBL- EBI	European Molecular Biology Laboratory-European Bioinformatics Institute
ERIC-PCR	enterobacterial repetitive intergenic consensus-based PCR
etc	et cetera
g	Gram
G	guanine

HPS	*Haemophilus parasuis*
IACUC	Institutional Animal Care and Use Committee
IHA	indirect hemagglutination
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Milliliter
MLEE	Multi-locus enzyme electrophoresis
mm	Milimeter
mM	Millimolar
mPCR	Multiplex Polymerase Chain Reaction
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
ng/μL	Nanogram per microliter
ng/reaction	Nanogram per reaction
NJ	Neighbour-joining
NT	Non-typeable
NTC	No template control
OMP	Outer membrane protein
OMPP2	Outer membrane protein P2
OMPP5	Outer membrane protein P5
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PCV2	Porcine circovirus type 2
PRDC	Porcine Respiratory Disease Complex

PRRSV	Porcine reproductive and respiratory syndrome virus
PFGE	Pulsed field gel electrophoresis
rep-PCR	Repetitive element based-polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
sec	Second
TAE	Tris-acetate-ethylenediaminetetraacetic acid
UK	United Kingdom
UPM	Universiti Putra Malaysia
USA	United States of America
UV	Ultraviolet
v	Version
vtaA	Virulence associated trimeric autotransporter
× *g*	Relative centrifugal force

CHAPTER 1

INTRODUCTION

In Malaysia, the pig industry is nearly self-sustainable and has remained stagnant for the past few years amounting at around 1.4 million head of pig production each year (Malaysia Department of Veterinary Services, 2018). A slight drop of pig production was observed in the recent three years due to the closing of numerous small-scale farms. Farms of Peninsular Malaysia account most of the Malaysia's pig production in which farms in Sarawak also contributed a significant number.

Swine respiratory diseases are common among pigs especially in the weaners group (Oliveira, 2002). Piglets are exposed to the respiratory pathogens as early since the first week of age and cause high mortality during outbreaks (Choi et al., 2003; Turni and Blackall, 2007). Porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) are few viruses affecting the respiratory system of pigs and is often complicated with secondary bacterial infections such as *Haemophilus parasuis, Mycoplasma hyopneumoniae* and *Streptococcus suis* (Thacker, 2001; Kim, Chung and Chae, 2003; Hansen et al., 2010; Opriessnig et al., 2011). As a matter of fact, appearing as a complex of multiple agents causing respiratory problem in pigs, it is termed as porcine respiratory disease complex (PRDC).

Commercial vaccines have been widely developed to target porcine viral diseases and have proved to be effective in controlling diseases. On the other hand, with the exception of *M. hyopneumoniae*, researchers still face hardship in developing the most ideal vaccines for swine bacterial diseases due to their vast genotypic and phenotypic variability (Dekker et al., 2012; Liu et al., 2016). In Malaysia, neither active study is being carried out nor a proper diagnostic tool that fully differentiating each bacterial pathogen in pigs is available. Most of the time, diagnosis was based on presumptive observation of post mortem lesions which most of the time might not be true.

Important bacterial pathogens of swine were often difficult to isolate (Olvera et al., 2007; Cook et al., 2016). Without proper sampling conditions and appropriate culture methods, the suspected disease would have been misdiagnosed. *Haemophilus parasuis* is one important disease of the swine and is ubiquitous (Miani et al., 2017). Not only causing Glässer's disease on its own, it also contributes to the PRDC, which is one of the leading cause for the mortality in grower and finisher pigs (Aragon et al., 2012; Howell et al., 2015). Glässer's disease is present in all major pig farming countries and the prevalence is increasing (Aragon et al., 2012). Nonetheless, Malaysia having its own pig population is not exempted from this issue. Over the fifty years of pig farming in Malaysia, Glässer's disease has persisted and yet efforts in

defining this bacteria is absent and strategies to control this disease often come to no avail.

Haemophilus parasuis is a pathogen of great economic impact. Based on United States statistic, it is the second major causes of mortality in prewean pigs (Holtkamp, Rotto, and Garcia, 2007). Infection rate can go up to 50-75% and mortality rate ranges above 10% (Bak and Riising, 2002; Aragon et al., 2012). Nowadays, it was not only common in the weaner pigs but also the grower pigs. This is a significant loss to the production due to the great number of mortality and unthriftiness (Castilla et al., 2012). This disease is currently considered a menace to the industry since high cost of antimicrobial usage are inflicted and affected animals are disposed, and this occurs even in farms with good husbandry management (Nedbalcova et al., 2006). According to the National Animal Disease Information Service (NADIS) of UK, losses in an outbreak farm were estimated at £14000 in a 300-sow farm per year alone (White, 2012). Therefore, effective and time saving methods of pathogen detection were extremely crucial in disease management. Prompt diagnosis will prevent further disease spread and minimize losses from the negative impacts of disease.

Currently, the available commercial *H. parasuis* vaccines are serotype-based. Undesirable results of vaccination were seen in some farms, providing the idea that vaccine strains are heterologous of field strain and the immunity triggered was not sufficient for protection (Liu et al., 2016). Although cross-protection among serotypes has been portrayed, yet complete protection is still impossible as the neutralizing antibodies production against heterologous strains were low (Miniats, Smart and Rosendal, 1991; Nielsen, 1993; Rapp-Gabrielson et al., 1997; Bak and Riising, 2002). Therefore, the knowledge of the serotype distribution is indeed of paramount importance for the best vaccine selection for disease control. In long term, it is hope that the *H. parasuis* can be effectively control via strategic vaccination in turn minimizing disease occurrence; ultimately reducing the dependent of antimicrobial in disease management.

Despite frequent occurrence of *H. parasuis* in Malaysia pig herds, the nature of these bacteria remains unknown. Therefore, the objectives of this study are:

1. to detect presence of *H. parasuis* in Peninsular Malaysia clinical samples/pigs using PCR technique.
2. to detect major serotypes of *H. parasuis* namely serotype 4, 5 or 12 and 13 in using multiplex PCR assay.
3. to detect the *OMPP2* gene of *H. parasuis* using PCR technique.
4. to determine the phylogenetic relationship of Peninsular Malaysia *H. parasuis* and other reference strains by *OMPP2* virulence gene.

It is hypothesize that:

1. *Haemophilus parasuis* is present in Peninsular Malaysia and is detectable by using PCR technique.
2. serotypes 4, 5 or 12 and 13 are present in Peninsular Malaysia.
3. *OMPP2* gene is detected in *H. parasuis*.
4. there is a close relationship between Peninsular Malaysia *H. parasuis* and other reference strains.

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